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Discovery

Kinetics of partially purified alpha-amylase produced by *Brevibacillus borostelensis* R1

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ABSTRACT

Amylolytic enzymes have great biotechnological applications and economic exploitations. The partially purified α-amylase from *Brevibacillus borostelensis* R1 showed highest activity at 37^{0}C and pH 7.0 (6133±58U/ml). The Vmax (17.73±0.5612 Uml^{-1}) was observed at starch concentration (3.5mg) and the Km value was 1.368±0.1292mg/ml and the amount of α-amylase activity was found to be Kcat (7.092±0.2245mg/ml). There was a linear relationship between the α-amylase activity and substrate concentration. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight of the enzyme at pH 8.3 in 12% slab gel. A single protein band obtained indicates monomeric nature of the enzyme. The molecular weight of the enzyme was found to be 43kDa. Specific staining was conducted to confirm the protein band as α-amylase. Alpha-amylases have several applications in desizing of textiles, destaining the stains, improvement of shelf life of bread, dual production of ethanol, effluent treatment, sewage treatment and other fermentation processes.

Keywords: Alpha-amylase, Kinetics, *Brevibacillus borostelensis* R1

1. INTRODUCTION

The rate of an enzyme catalyzed reaction increases with rise in temperature till it reaches the maximum. The effect of temperature is due to thermal stability of enzyme, alteration of pK_as by the heat of ionization, affinity of an enzyme for the activators or inhibitors, different temperature coefficients and change in rate limiting functions (Sawhney & Randhir Singh, 2001). The α-amylase activity with optimum temperature range 30^0C-50^0C was reported in *Bacillus spp.* (Kusuda et al., 2003). The optimum temperature range, 55^0C-70^0C for alpha-amylase activity was also reported in *Bacillus spp.* (Deutch, 2002). Extreme optimum temperature for amylase activity range 75^0C-100^0C was reported in *Bacillus spp.* (Burhan et al., 2003). Distribution of the total enzyme among the various ionic forms depends on the pH and the ionization constants of the various groups. Since the catalytic activity is usually confined to a narrow range of pH, only one of the ionic forms of the enzyme is catalytically active. The optimal pH of α-amylase activity around acidic range 3.0-6.5 was reported in *Bacillus spp.* (Gupta et al., 2003). Several authors reported a neutral pH of 7.0 for amylase activity from *Bacillus spp.* (Tanyildizi et al., 2005). The optimal pH of amylase activity at alkaline range of 8-12 was reported in *Bacillus spp.* (Burhan et al., 2003). The rate of an enzyme depends on the amount of substrate available. At relatively low substrate concentration, initial velocity increases linearly with an increase in the substrate. At higher substrate concentration, initial velocity increases smaller extent in response to increase in substrate. Finally a point is reached beyond which there is no increase in the velocity with increase in the concentration plateau is called the Vmax (Sawhney & Randhir Singh, 2001). Several authors reported Km value of α-amylase in *Bacillus spp.* by using starch as substrate: *Bacillus subtilis* (Macro et al., 1996) and *Bacillus flavothermus* (Boltan et al., 1997). The molecular weight of α-amylase range 10-50 kDa was reported in *Bacillus spp.* (Alva et al., 2007). The molecular weight of α-amylase range 51-100 kDa was reported in *Bacillus spp.* (Aguilar et al., 2000). The molecular weight of α-amylase range 101-210kDa was reported in *Chloroflexus aurantiacus* (Ikram-ul-haq et al., 2010).

2. MATERIALS AND METHODS

The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at 37ºC for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The absorbance was read at 546 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions (Miller, 1959).

The effect of temperature on α-amylase activity was checked by adding 0.5 ml of starch (0.5% in phosphate buffer at pH 6.8) to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37^0C. To this mixture partially purified enzyme (0.5ml) was added and incubated at different temperatures 4^0C, 20^0C, 25^0C, 37^0C, 50^0C, 60^0C and 70^0C for 15 minutes. One milliliter of 3, 5-dinitrosalicylic acid reagent (1%) was added to stop the reaction. Estimation of α-amylase activity was carried out according to DNS method.

The effect of temperature stability of α-amylase activity was tested by adding 0.5 ml of starch (0.5% in phosphate buffer at pH 6.8) to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37^0C. Partially purified enzyme (0.5ml) was added and incubated at different temperatures 4^0C, 20^0C, 25^0C, 37^0C, 50^0C, 60^0C and 70^0C for different hours (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). For control the partially purified amylase was incubated at 37^0C for 15 minutes. One milliliter of DNS reagent (1%) was added to stop the reaction.

The effect of pH on α-amylase activity was assayed. Different pH buffers (0.1ml) with 2.0-11.0 were prepared- Hydrochloric acid/ Potassium chloride pH (1.0-2.2), sodium acetate pH (3.0-4.0), sodium citrate pH (5.0-6.0), Tris-HCl pH (7.0-8.0), Glycine-NaOH pH (9.0-11.0). The 0.5ml of starch (0.5% w/v was taken from different aliquots prepared from varying pH) added to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37^0C. Partially purified enzyme (0.5ml) was added and incubated at 37^0C for 15 minutes. One milliliter of DNS reagent (1%) was added to stop the activity after incubation.

The α-amylase activity was assayed by adding 0.5ml of different starch concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, and 5.0% in phosphate buffer at pH 6.8) to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37^0C. Partially purified enzyme (0.5ml) was added and incubated at 37^0C for 15 minutes. One milliliter of DNS reagent (1%) was added to stop the reaction. The substrate concentration was expressed in [S] (mg) and estimation of α-amylase activity V (U ml^{-1}) was carried out according to the dinitro salicylic acid (DNS) method. The Km and Vmax of the reaction were determined by processing the data and drawing Michaelis-Menten (v vs [S]) plot.

The sample protein was mixed with an equal volume of sample 2X buffer [12.5ml Tris-HCl (1M), pH 6.8, 4.0g SDS, 10.0ml β-mercaptoethanol, 20.0ml glycerol, 4.0ml Bromophenol blue (1%) to make final volume to 100ml] and boiled for 3min in a boiling water bath and cooled to room temperature (Sawhney & Randhir singh, 2001). SDS-PAGE (12%) was carried out for determination of molecular mass (Laemmli, 1970). A slab gel with 20.0ml 5% staking gel [3.4ml acrylamide-bis-acrylamide (30:0.8), 2.5ml stacking gel buffer stock solution (1.0M Tris-HCl, pH 6.8), 0.20ml 10% of SDS, 0.02ml 10.0% APS (freshly prepared), 13.6ml of H_2O, 0.02ml TEMED] and 30.0ml 12% resolving gel [12.0ml acrylamide-bis-acrylamide (30:0.8), 7.5ml resolving gel buffer stock solution (1.5M Tris-HCl,

pH 8.8), 0.30ml 10% SDS, 0.30ml 10.0% APS, 9.90ml H_2O, 0.012ml TEMED], freshly prepared running buffer [3.0g Tris, 14.4g glycine, 1.0g SDS (pH 8.3)] were used. Partially purified sample and molecular weight marker protein samples (20µl) were loaded with micropippte in the wells. The DC power was supplied (15mA, 200V) for initial 15min until the sample had travelled through the stacking gel. Later the current was increased (30mA, 500V) until the bromophenol blue dye (0.01%) reached the bottom of the gel slab.

All the experiments were conducted in triplicate. The results were given as mean value ± standard deviation. The conditions were analyzed to determine the significant difference between the variables by one way ANOVA, two way ANOVA and correlation analysis by using the scientific graph pad (Prism 6.1version software). Analysis of variance (ANOVA) refers to the examination of differences among the sample means. It is used to examine the significance of the difference amongst more than two sample means at the same time.

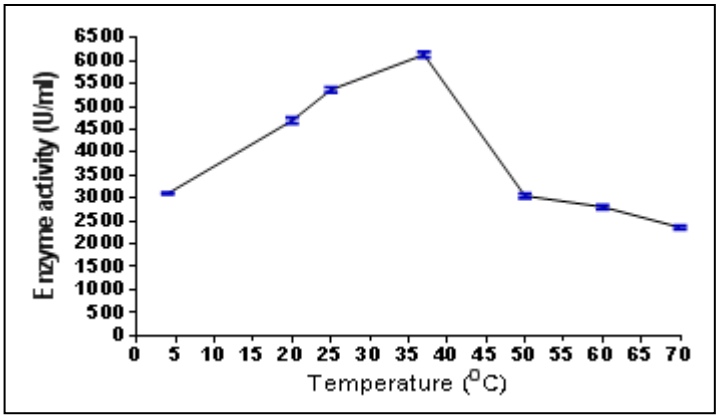

Figure 1

Optimum temperature of partially purified α-amylase; Y bars indicate the standard deviation of mean value.

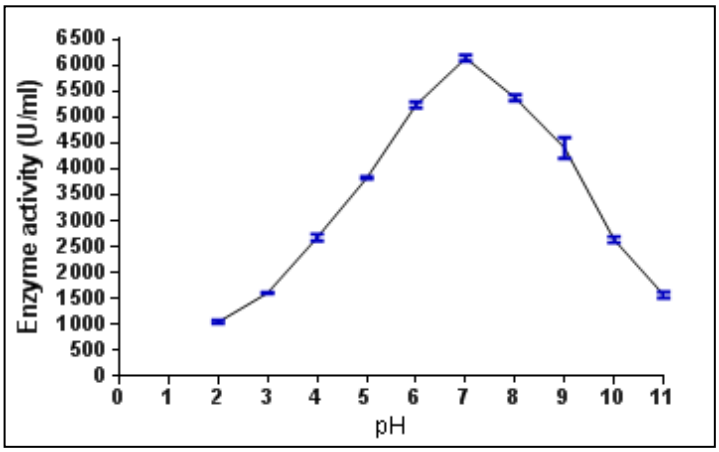

Figure 2

Optimum pH of partially purified α-amylas; Y bars indicate the standard deviation of mean value.

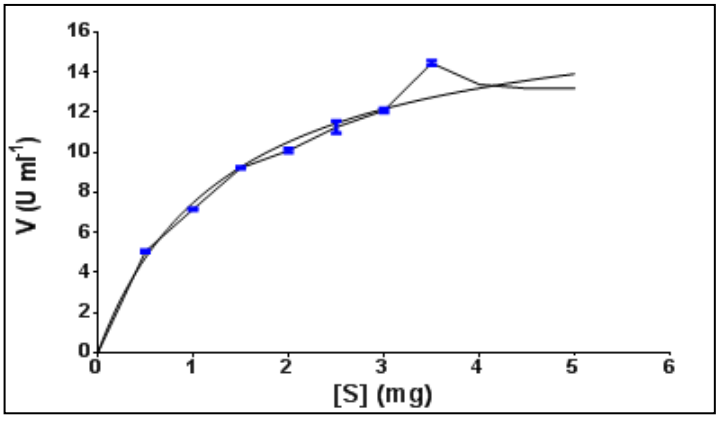

Figure 3

Effect of substrate concentration on the activity of α-amylase by Michaelis-Menten plot

3. RESULTS

The highest amylase activity was observed at 37^{0}C (6133±58U/ml), lowest at 70^{0}C (2360±40U/ml). Only 38.47% activity remained at 70^{0}C (Figure 1). The optimum temperature of partially purified enzyme was similar to the crude enzyme. As shown in Figure 2, the highest amylase activity was observed at pH 7.0 (6133±58U/ml), lowest at pH 2.0 (1047±31U/ml). The amylase activity of the partially purified enzyme increased up to pH 7.0 and decreased from then onwards. The optimum pH of partially purified enzyme was similar to the crude enzyme. The rate of catalytic activity of partially purified α-amylase was studied by Michaelis-Menten equation. It is the rate equation for reactions catalyzed by enzymes having a single substrate. Katal (Kcat) is defined as the amount of enzyme activity that transforms one mole per second of substrate. Starch was used as substrate (0.5 to 5.0 mg) to study the kinetics of amylase activity. The Vmax (17.73±0.5612Uml^{-1}) was observed at starch concentration (3.5mg) and the substrate concentration at 1/2Vmax was Km (1.368±0.1292mg/ml) and the amount of enzyme activity was found to be Kcat (7.092±0.2245mg/ml). At 95% confidence intervals the Vmax (16.59 to 18.87Uml^{-1}), Km (1.104 to 1.631 mg/ml) and Kcat (6.634 to 7.550mg/ml) are shown in Figure 3. As the substrate (starch) concentration increased a linear relationship between amylase activity and substrate was observed. SDS-PAGE (Polyacrylamide gel electrophorosis) was conducted to determine molecular weight at pH 8.3 in 12% slab gel (Figure 4). The first lane was loaded with molecular weight markers: phosphorylase (97kDa), Bovine serum albumin (67kDa), Ovalbumin (43kDa), Carbonic anhydrase (29kDa), Soybean trypsin inhibitor (20kDa) and Lysozyme (14kDa).The second lane was loaded with partially purified enzyme. The molecular weight of the α-amylase was found to be 43kDa. The protein band showed in Figure 4 was single indicating monomeric nature of the enzyme.

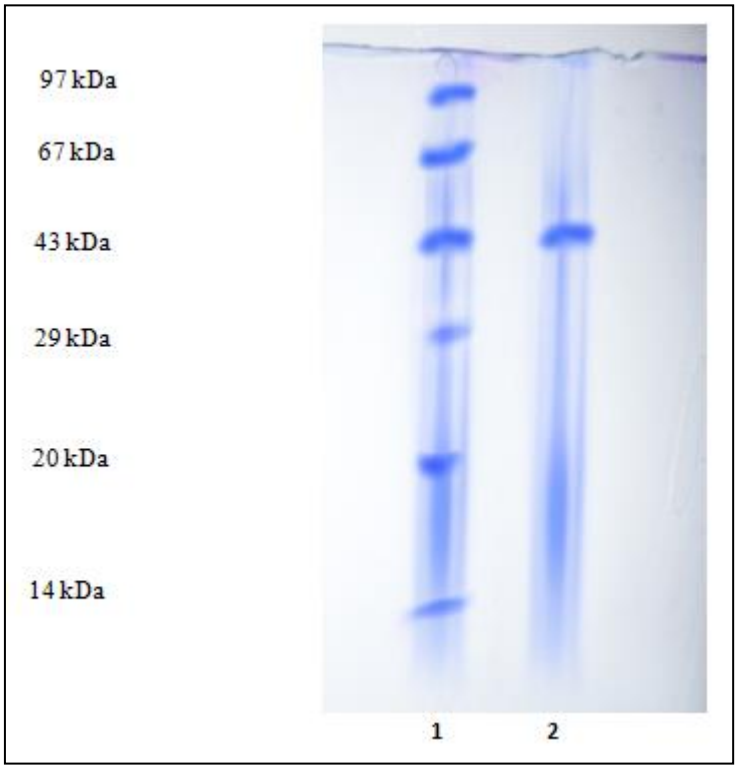

Figure 4

Determination of molecular weight of α-amylase isolated from *Brevibacillus borstelensis* R1 using SDS-PAGE

Lane 1. Molecular weight markers: 97kDa (phosphorylase), 67kDa (Bovine serum albumin), 43kD (Ovalbumin), 29kD (Carbonic amnhydrase), 20kDa (Soybean trypsin inhibitor), 14kDa (Lysozyme).

Lane 2. Partially purified fraction.

4. DISCUSSION

The optimum temperature of partially purified α-amylase of *B.borostelensis* R1 was studied. The highest amylase activity was observed at 37^{0}C (6133±58U/ml). Only 38.47% activity remained at 70^{0}C. The optimum temperature of partially purified enzyme was similar to the crude enzyme. Optimum temperature range (30-50^{0}C) was reported in *Halomonas meridian* (Coronado et al., 2000). The optimum pH of partially purified α-amylase was 7.0. Similar reports were given by Malhotra et al. (2000), Haq et al. (2002) and Haq et al. (2005) in *Bacillus* sps. The effect substrate on the rate of catalytic activity of partially purified α-amylase was studied by Michaelis-Menten equation. The Vmax (17.73±0.5612 Uml^{-1}) was observed at starch concentration of 3.5mg/ml. The Km value of the enzyme was (1.368±0.1292mg/ml). The Km values of partially purified amylases were reported to be within the range of 0.35 to 4.7mg/ml. Several authors reported Km value of α-amylase by using starch as substrate: 11.176 mg/ml/h in thermophilic *Bacillus subtilis* strain (Konsula & Liakopoulou-Kyriakides, 2004) and 600milliunits/mg in *Bacillus acidocal-darius* strain RP1 (Natalia et al., 2006). Hanumanthu Prasanna Lakshmi (2013) reported Km (11.57±0.409mg/ml), Vmax (0.032±0.0036 (mg/ml/min) and Kcat (1.6517±0.055mg/ml) in *Staphylococcus aureus.* Amico et al. (2002) reported Km (234 µM) of amylase in *Pseudoalteromonas haloplanktis* using 3.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4, 6-O-ethylidene as substrate. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% slab gel was run at pH 8.3 with molecular weight markers. Our findings suggested that the quaternary structure of the α-amylase of the *Brevibacillus borostelensis* R1 was monomeric with 43kDa molecular weight. Approximate molecular mass (42kDa) was reported by Alva *et al.* (2007). In *Bacillus cereus* GUF8 (Aguilar *et al*., 2000) purified α-amylase appeared as a single polypeptide with a molecular weight of about 56kDa similar to *Bacillus licheniformis* and *Bacillus amyloliquefaciens* α-amylases.

5. CONCLUSION

The highest α-amylase activity was observed at 37^{0}C (6133±58U/ml), while the lowest at 70^{0}C (2360±40U/ml). Only 38.47% activity remained at 70^{0}C. The highest α-amylase activity was observed at pH 7.0 (6133±58U/ml), and the lowest at pH 2.0 (1047±31U/ml). The rate of catalytic activity of partially purified α-amylase was studied by Michaelis-Menten equation. Starch was used as substrate (0.5 - 5.0 mg/ml) to study the kinetics of α-amylase activity. The Vmax (17.73±0.5612 Uml^{-1}) was observed at starch concentration (3.5mg) and the Km value was 1.368±0.1292mg/ml and the amount of α-amylase activity was found to be Kcat (7.092±0.2245mg/ml). There was a linear relationship between the α-amylase activity and substrate concentration. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight of the enzyme at pH 8.3 in 12% slab gel. A single protein band obtained indicating monomeric nature of the enzyme. The molecular weight of the enzyme was found to be 43kDa. Specific staining was conducted to confirm the protein band as α-amylase.

SUMMARY OF RESEARCH

1.The partially purified α-amylase from *Brevibacillus borostelensis* R1 showed highest activity at 37^{0}C and pH 7.0 (6133±58U/ml).

2.The Vmax (17.73±0.5612 Uml^{-1}) was observed at starch concentration (3.5mg) and the Km value was 1.368±0.1292mg/ml and the amount of α-amylase activity was found to be Kcat (7.092±0.2245mg/ml). There was a linear relationship between the α-amylase activity and substrate concentration.

3.Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight of the enzyme at pH 8.3 in 12% slab gel. A single protein band obtained indicates monomeric nature of the enzyme. The molecular weight of the enzyme was found to be 43kDa.
4.Specific staining was conducted to confirm the protein band as α-amylase.

FUTURE ISSUES

Alpha amylase produced by *Brevibacillus borostelensis R1* was characterized and the kinetic behavior of the enzyme was studied to continue this if the amino acid sequence is done it would yield better understanding of the applications of the enzyme.

DISCLOSURE STATEMENT

There is no special financial support for this research work from the funding agency.

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